A Molecular Fingerprint Technique to Detect Pollution-Related Changes in River Cyanobacterial Diversity

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ABSTRACT

Humans now have a strong influence on almost every major aquatic ecosystem, and our activities have dramatically altered the quality of receiving waters worldwide. Thus, there is a continuous need to develop and apply novel and effective technologies to detect, manage, and correct human-induced degradation of aquatic systems. In the present work, we evaluated the molecular approach using polymerase chain reaction (PCR)-temperature gradient gel electrophoresis (TGGE) to measure changes in cyanobacterial diversity along a pollution gradient in a river and compared it with that of using microscopic observations of field-fixed and cultured samples. The different 16S rDNA genes present in the cyanobacterial community of each sampling point of the river were separated by TGGE, giving a characteristic pattern of bands for each site. This pattern represents a "fingerprint" of the community, allowing direct comparisons of the different samples. The TGGE results revealed that the structure of the cyanobacterial community differed along the pollution gradient of the river. Microscopic and molecular approaches showed that cyanobacterial diversity decreased in a downstream direction. Similar results were obtained by the two methods, as indicated by the high correlation between them. We suggest PCR-TGGE could be a useful and rapidly applied technique for the routine analysis of changes in cyanobacterial diversity in response to pollution, which would allow us to monitor rivers in surveillance networks of watercourse quality.

Many rivers are greatly influenced by anthropogenic factors and disturbance due to pollution, and specific problems arise from other types of human interference. Humans now strongly influence almost every major aquatic ecosystem, and our activities have dramatically altered the fluxes of growth-limiting nutrients from the landscape to receiving waters. These nutrient inputs have had profoundly negative effects on the quality of receiving waters worldwide (Correl, 1998; Smith, 2003). Multiple negative ecological impacts from agriculture, industry, urban development, and recreation have been reported (Smith, 2003; Power et al., 2005). Sources of nutrients, pesticides, metals, gasoline-related compounds, and other contaminants represent major challenges that continue to affect streams and ground water (Power et al., 2005; USGS, 2006).

There is a need to monitor the status and trends in freshwater quality to quantify the impacts of human actions on freshwater systems and to improve freshwater biodiversity conservation. Historically, many countries have assessed river water quality simply in terms of the chemical or pollution status of the water flowing in river channels. However, a more comprehensive view of river habitats is needed to answer pressing ecological questions such as those arising from the EC Water Framework Directive (WFD) (European Commission, 2000). It is now imperative that we develop and apply novel and effective technologies to detect, manage, and correct human-induced degradation of aquatic systems. The great financial investment in freshwater management means that decisions are often based on biomonitoring results (Bonada et al., 2006). Among aquatic biota, microorganisms are generally highly sensitive to and profoundly affected by environmental perturbations (Paerl et al., 2003), and cyanobacteria, because of their position at the base of aquatic food webs, play an especially significant role in freshwater use and management.

Our previous studies have demonstrated changes in the structure of cyanobacterial communities in response to pollution in watercourses (Perona et al., 1998; Douterelo et al., 2004; Perona and Mateo, 2006). We found that cyanobacterial diversity decreased with increasing pollution in several rivers and streams. At polluted sampling sites, the number of species declined, but there was an increase in the overall cyanobacterial abundance caused by the proliferation of a few well adapted species (Douterelo et al., 2004). However, studies of cyanobacterial diversity and community structure have traditionally been based mainly on the accurate taxonomic identification of the individuals found. This approach is often time-consuming due to difficulties identifying species that exhibit extreme morphological variability and whose taxonomy is controversial (Komárek and Anagnostidis, 1999). Moreover, in many cases, the only means of identification is through the comparison of live material with cultured field samples, which introduces more difficulties due to culture selectivity and the fact that many species frequently develop anomalous morphological states in culture (Komárek and Anagnostidis, 1999; Perona et al., 2003). Consequently, several authors have drawn attention to the need to develop new approaches based on ultrastructural, biochemical, and molecular information (Whitton, 1992; Komárek and Anagnostidis, 1999).

Molecular approaches have been developed for the detection and characterization of bacteria in the environment (Amann et al., 1995). Temperature gradient gel electrophoresis is a PCR-based tool that has been used to analyze the diversity of bacterial assemblages in different environments (Fromin et al., 2002; Valentin et al., 2005) In the present work, the PCR-TGGE-based analysis of changes in cyanobacterial diversity along a pollution gradient in a river was tested and compared

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Abbreviations: PCR, polymerase chain reaction; TGGE, temperature gradient gel electrophoresis; SRP, soluble reactive phosphate; DGGE, denaturing gradient gel electrophoresis.

with microscopic observations of field-fixed and cultured samples. In our analysis, the different 16S rDNA genes present in the cyanobacterial community of each sampling point of the river were discriminated by TGGE, giving a characteristic pattern of bands for each site. This pattern represents a "fingerprint" of the community, allowing direct comparisons among different samples. Our study aimed to establish whether similar results were found by both methods, since, if this were so, it would allow us to develop a standard molecular cyanobacteria-based technology for monitoring rivers in surveillance networks of watercourse quality.

MATERIALS AND METHODS

Sampling Sites and Sampling Procedure

The samples used in this study were collected from the Guadarrama River, located in central Spain, near the city of Madrid. Sampling sites were selected to include locations above and below populated areas. Sampling site 1 (UTM 30TVL4500407) is located in an unaffected by human influence area, upstream of an industrial and populated zone, site 2 (UTM30TVK4486420) and site 3 (UTM30TVK4474419), 22 km and 38 km downstream, which receive industrial and domestic sewage from the nearby human settlements.

Dissolved oxygen and conductivity were measured in situ at every sampling site using a YSI 6920 Multiparametric Probe (YSI, Baton Rouge, LA) set up on the river bank. Measurements were made at each sampling site at the same time as the water samples were collected, including higher running velocity locations and more marginal, slower velocity locations. Water samples for chemical measurements were collected in polyethylene bottles and kept cool in the dark. Three stones were collected haphazardly at 0.1- to 0.3-m depth on the vertical, from a submerged part of the riverbank at each sampling location. The attached cyanobacteria were removed by brushing an area of 16 cm^2 of the stone. Detailed information about preservation, cultivation, and microscopic examination are provided in Perona et al. (1998) and Perona et al. (2003). Aliquots for DNA extraction (see below) were frozen.

Nutrient chemical concentrations (NH_4^+–N, NO_3^-–N, NO_2^-–N, PO_4^{3-}–P) were determined in situ using colorimetric methods, adapted from Standard Methods for the Examination of Water and Wastewater (APHA, 1992), using a DREL-2010 portable laboratory (Hach Company, USA). Ammonium nitrogen was measured by the Nessler method (estimated detection limit 0.05 mg L^{-1}, precision ± 0.015 mg L^{-1}), reading the absorbance at 425 nm. When values were close to the detection limit, the salicylate method was used (detection limit 0.01 mg L^{-1}), reading absorbance at 655 nm. The NO_3^-–N was analyzed using a modification of the cadmium reduction protocol, using gentisic acid instead of 1-naphthylamine (estimated detection limit 0.5 mg L^{-1}) and reading absorbance at 500 nm. Samples below that detection limit were measured by a low-range method, which is an expanded modification of the former one, using a chromotropic acid indicator (estimated detection limit 0.05 mg L^{-1}, precision ± 0.01 mg L^{-1}) and reading at the same wavelength. The NO_2^-–N was determined colorimetrically using chromotropic and sulphanilic acids as indicators (estimated detection limit 0.01 mg L^{-1}, precision ± 0.01 mg L^{-1}) and reading absorbance at 500 nm. The soluble reactive phosphate (SRP = PO_4^{3-}–P) was measured by a modification of the molybdenum blue procedure (estimated detection limit 0.01 mg L^{-1}, precision ± 0.01 mg L^{-1}) reading absorbance at 890 nm. Every nutrient analysis was performed three times. The standard deviations of the pseudoreplicates remained within the typical precision range for each method. Cyanobacteria were identified using standard light, phase-contrast, and epifluorescence microscopy. Their abundance in epilithon samples was evaluated by counting the presence of each species using a Neubauer chamber.

DNA Extraction and Purification

DNA was extracted from 1.5-mL aliquots of cell suspensions of each extracted biofilm sample.

The DNA extraction protocol used in this study was derived from that of Porteous et al. (1997). Cyanobacterial cells were lysed by a hot extraction procedure performed with 4% sodium dodecyl sulfate, 250 m*M* NaCl, 100 m*M* EDTA, and 350 m*M* guanidine isothiocyanate at 68°C, after vortex mixing for 1 min and sonication for 2 min. After incubation the suspension was centrifuged for 15 min at 13 000 *g* (Eppendorf Minifuge), and 600 μL of the supernatant phase was mixed with 75 μL 5 *M* potassium acetate, 250 μL 40% polyethylene glycol 8000, and 10 μg of glycogen. Crude DNA extracts were partially cleaned using hexadecyltrimethylammonium bromide (CTAB), chloroform extraction, isopropanol precipitation and filtration with Microcon 100 microconcentrators (Amicon, Beverly, MA, USA). The size and quality of the extracted DNA was visualized on agarose gels after ethidium bromide staining and UV transillumination (Gel Doc 1000; BioRad Laboratories; Hercules, CA, USA).

Polymerase Chain Reaction Amplification

The polymerase chain reaction of 16S rRNA was performed using specific oligonucleotide primers for cyanobacteria (Nübel et al., 1997): CYA 359F (with a 20-nucleotide GC clamp at the 5' end) and CYA 781R. Thermocycling was performed using a PerkinElmer Gene Amp Thermocycler, which gave PCR products of about 450 bp in length. Denaturation was performed at 94°C for 5 min, followed by 35 incubation cycles, each of 94°C for 1 min, 60°C for 1 min, 72°C for 1 min, and a final extension step at 72°C for 9 min. The PCR products were stored at 4°C.

Temperature Gradient Gel Electrophoresis Analysis

Separation of DNA fragments by TGGE is based on the decreased electrophoretic mobility of partially melted double-strand DNA molecules in denaturing polyacrylamide gels through a linear temperature gradient. Molecules with different sequences may display different melting behaviors and will therefore migrate at different positions in the gel.

Polymerase chain reaction amplification products were directly used for TGGE analysis. Temperature gradient gel electrophoresis (Maxi TGGE Biometra, Germany) was performed by loading 6 μL of PCR product in a gel consisting of 5% acrylamide/bisacrylamide, 1% TAE, 7 *M* urea, 20% formamide, and 2% glycerol. The electrophoresis was run in 1 × TAE for 16 h at 130 V with a gradient ranging from 42 to 54°C. The gel was then stained following a routine silver-staining protocol (Heuer et al., 1997). Digital images of the gels were analyzed using the Quantity One software package (BioRad Laboratories; Hercules, CA, USA). Temperature gradient gel electrophoresis banding patterns were computer-assisted characterized as described by Fromin et al. (2002): After background subtraction and normalization, bands were described by its standardized position (*Y*, in pixel, on the image file), and its relative intensity in the profile(P_i), which is calculated by the relative surface of the peak in the profile ($P_i = n_i/N$, where n_i is the surface of the peak *i*, and *N* is the sum of the

surfaces for all the peaks within the profile). The data obtained, relative abundances and positions was used for further statistical analysis.

Diversity Analysis

Diversity was measured using the Shannon-Weaver Index (Shannon and Weaver, 1963): $H = -\sum p_i \log_2 p_i$, where p_i is the proportion of each taxa (microscopic analysis) or proportion of each band in the TGGE gels as above described. Differences in the means were investigated by one-way ANOVA, using SPSS version 11.0 (SPSS, 2002).

RESULTS AND DISCUSSION

Differences in water quality were found along the stretch of the river studied (Table 1). A gradual decrease in dissolved O_2 in a downstream direction was observed, while conductivity increased with the increase of dissolved chemical species, including nutrients. Values of nutrient variables (dissolved inorganic nitrogen and soluble reactive phosphate) were considerably higher at downstream than at upstream sampling sites. Greater amounts of soluble reactive phosphate were detected at sites 2 and 3, which can probably be attributed to domestic waste sewage. Downstream sections have been heavily affected by human activities and exhibit a clear pollution gradient of nutrient enrichment. Changes in the structure of the cyanobacterial community were also found, whereby species richness, estimated by microscopic analysis, clearly differed among the sampling sites along the river (Fig. 1). A marked decrease in the number of species was observed in polluted downstream sites. The reduction in species richness in the lower locations was mainly due to a decrease in the number of heterocystous species, although the number of non-heterocystous species also decreased at site 3 (Fig. 1). Heterocystous cyanobacteria, which are able to fix N_2, are probably better adapted to a low-nitrogen medium but have a lower competitive ability than do non-heterocystous species in nitrogen-enriched media such as downstream locations (Fig. 1). A similar pattern was found in previous studies of other rivers based on microscopic and cultivation techniques (Perona et al., 1998; Douterelo et al., 2004). Other studies in polluted waters of tropical rivers also showed changes in the relative proportions of non-heterocystous and heterocystous species (Branco and Pereira, 2002). Thus, there was a general trend in more polluted sites toward reduced species richness, and a dominance of N-fixing cyanobacteria in waters with lower levels of N.

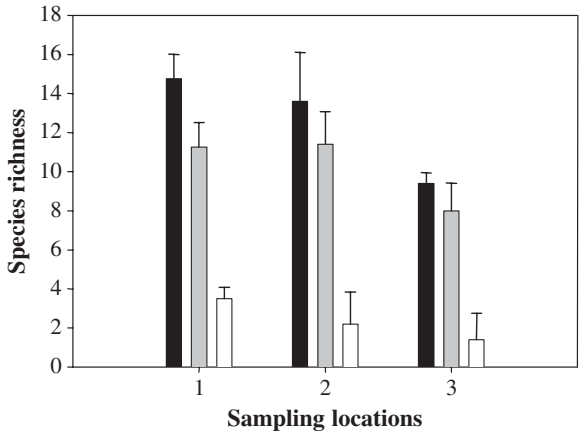

Fig. 1. Species richness (SR) at sampling locations based on microscopic examination; black bar, overall SR; gray bar, non-heterocystous species; white bar, heterocystous species.

The values of the Shannon-Weaver Diversity index (Table 2) also diminished progressively downstream. Statistically significant differences were found between sites 1 and 2, and between sites 1 and 3 ($p < 0.05$).

The TGGE analysis showed noticeable differences in the banding patterns at the various sampling locations (Fig. 2). The number of bands was similar among replicates (different stones) from each location but clearly differed among the sites with distinct levels of pollution, whereby more bands were present in upstream than in downstream sites (Fig. 2). To compare the results of microbial diversity obtained by direct microscopy and the molecular method, the Shannon-Weaver Diversity index based on the number and relative intensities of the bands in the TGGE gel was calculated (Table 2). The diversity decreased in a downstream direction, and there was a statistically significant difference ($p < 0.05$) between sites 1 and 3. Similar results were found by both methods, as corroborated by the very high and significant correlation of the estimates of the Shannon-Weaver index values for the microscopic and molecular approaches ($r = 0.91$, $p < 0.01$).

The traditional techniques used in bacterial diversity analysis present a range of difficulties, especially when analyzing epilithon cyanobacteria in some rivers in which the thin and strongly attached biofilm is difficult to identify, as when it is brushed from the stone the filaments may become fragmented and the material may be broken up into individual cells. This makes it essential to culture the samples in solid media, which in turn makes these studies less practical due to the greater time

Table 1. Variations along the three sampling locations in dissolved O_2 (DO), conductivity, dissolved inorganic nitrogen (DIN = NO_3^-–N + NO_2^-–N + NH_4^+–N), soluble reactive phosphate (SRP), and sulfate (SO_4^{2-}).

Characteristic (units)	Sampling location mean (minimum, maximum)		
	1	2	3
DO (mg L^{-1})	9.6 (8.8–9.95)	7.25 (5.05–7.55)	5.83 (4.6–7.05)
Conductivity (μS cm^{-1})	57.1 (20–86.4)	328.5 (226.6–426.5)	363.5 (241.1–478.5)
DIN (mg L^{-1})	0.2 (0.17–0.24)	5.79 (4.13–7.65)	5.97 (4.39–7.27)
SRP (mg L^{-1})	0.24 (0.01–0.6)	1.83 (1.3–2.39)	2.2 (1.45–3.35)
SO_4^{2-} (mg L^{-1})	5.5 (0–9)	32 (23–41)	39.5 (37–44)

Table 2. Shannon-Weaver diversity indices for cyanobacterial communities at sampling locations based on microscopic cell counts and temperature gradient gel electrophoresis (TGGE) digital-image analysis.

	Shannon-Weaver index	
Sampling locations	Microscopic analysis	TGGE analysis
1	2.92 ± 0.03	2.94 ± 0.03
2	2.22 ± 0.04	2.11 ± 0.2
3	2.12 ± 0.01	1.55 ± 0.03

needed to obtain results. The molecular fingerprinting techniques avoid these problems and introduce greater objectivity in the discrimination of diverse taxonomical units. Furthermore, traditional techniques are time-consuming and labor-intensive, while molecular techniques are much faster and often more accurate. Other advantages that have been claimed for the latter approach are their ease of execution, reproducibility of results, reliability, and speed (Muyzer and Smalla, 1998).

In cyanobacteria, denaturing-gradient gel electrophoresis (DGGE) has been used to study changes in cyanobacterial communities in microbial mats exposed to different levels of oil pollution and petroleum-degradation processes (Abed et al., 2002; van Bleijswijk and Muyzer, 2004). However, the number of investigations concerning cyanobacterial diversity in lotic ecosystems (creeks, rivers, and streams) is still limited compared with those in lakes or marine habitats. This is partly due to their changeability, which is determined by the water flow. Thus, the complexity and variability of running water habitats have discouraged systematic investigation. In this study, we chose TGGE instead of DGGE since the gradient is built by the apparatus itself and highly controlled by the microprocessors, which increases the reproducibility of the experiments. The PCR-TGGE approach has enabled us to detect variations in cyanobacterial diversity associated with changes in water quality in fluvial systems. The study of epilithon cyanobacterial diversity along this fluvial system yielded very similar results with the classical and molecular approaches. Analysis of bacterial diversity using Shannon-Weaver diversity indices based on fingerprint profiles have been previously performed in hypersaline and Antarctic microbial mats (Nübel et al., 1999; Taton et al., 2003); changes in TGGE bands intensities have been interpreted as changes in the relative abundance of the particular 16S rRNA gene in β-proteobacteria in biofilms of polluted rivers (Brümmer et al., 2003). Casamayor et al. (2002) demonstrated by a combination of DGGE and fluorescence in situ hybridization (FISH) in a Lake in Spain, that shifts in the relative intensity of a band in the community profile correlated with changes in the abundance of the respective microorganisms, determined by FISH counts with specific probes. Thus, the analysis of epilithon samples via PCR-TGGE could represent a simple and quick procedure to obtain diversity values that are of use in comparative studies.

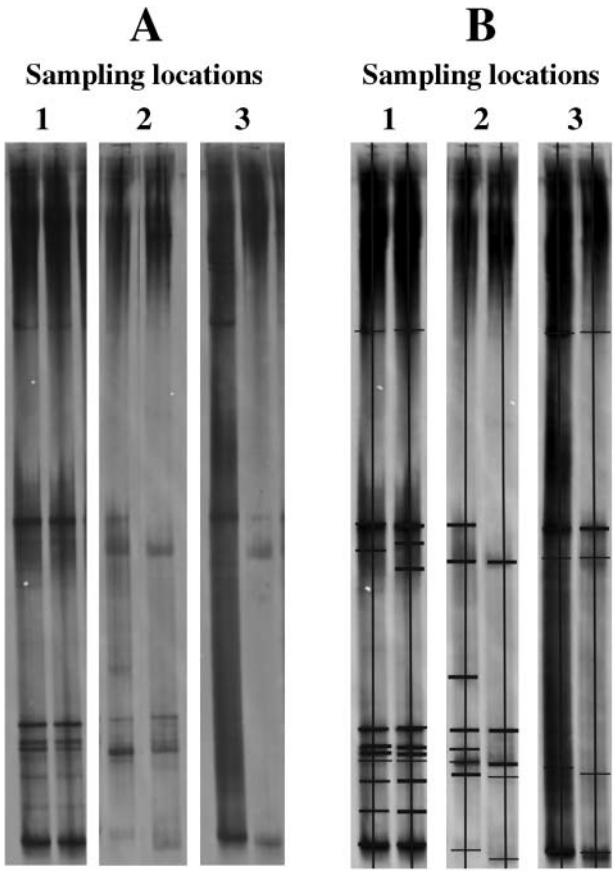

Fig. 2. Cyanobacterial community profiles based on specific amplification and temperature gradient gel electrophoresis (TGGE) separation of 16S rDNA fragments from duplicate, independent samples at each sampling location. (A) Original digital images. (B) Images analyzed to identify bands in each lane using the Quantity One program, (Bio-Rad).

The suitability of biological methods for monitoring environmental quality in aquatic environments, thereby supplementing established chemical analytical methods, has been discussed for a long time (Manly, 1996). Bioindicators are thought to be useful for a number of reasons. They may provide early warning of pollution or degradation of an ecosystem, alerting managers so that they can halt or mitigate its impact before critical resources are lost. They only assess pollutants that are thought to be important, i.e., those that are bioavailable and have adverse effects on biota (Maher and Norris, 1990). Unlike physical measures, which may only be useful for events with substantial impact, biological responses are cumulative and observable after the event that caused them. Thus episodic impacts become apparent through their lasting effects on the biota. Similarly, low-intensity, chronic impacts (e.g., low-level pollution) may be undetectable by physical or chemical measures while their cumulative biological effects may be easily detected. Bioindicators can help to assess synergistic or additive relationships between impacts, which is an important consideration in light of the typical combination of influences impinging on most ecosystems (Linton and Warner, 2003).

CONCLUSION

In conclusion, the results reported here support the view that once a community has been characterized and

its fingerprint obtained, this technique can be used to detect shifts in the diversity of cyanobacterial communities. Further studies identifying TGGE bands with the organisms in situ, selecting, cutting, and sequencing bands, and detailed analysis will reveal which species are responsible for the variation. However, if we merely adopted this approach with the aim of detecting pollution processes as rapidly as possible, the fingerprint characteristic of a community in a specific place could be used as a bar code that, like a detector or sentinel, acts as an 'early warning' device alerting us to the presence of pollutants in the environment. In this way, the technique could enable the efficient and rapid routine analysis of changes in cyanobacterial diversity in response to pollution, allowing us to monitor rivers in surveillance networks of watercourse quality more effectively.

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